Exhibit 6

Heat Shock Protein-Mediated Cross-Presentation of Exogenous HIV Antigen on HLA Class I and Class II[1]

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Strong CD4^{+} and CD8^{+} T cell responses are considered important immune components for controlling HIV infection, and their priming may be central to an effective HIV vaccine. We describe in this study an approach by which multiple CD4^{+} and CD8^{+} T cell epitopes are processed and presented from an exogenously added HIV-1 Gag-p24 peptide of 32 aa complexed to heat shock protein (HSP) gp96. CD8^{+} T cell recognition of the HSP/peptide complex, but not the peptide alone, was inhibited by brefeldin A, suggesting an endoplasmic reticulum-dependent pathway. This is the first report to describe efficient processing and simultaneous presentation of overlapping class I- and class II-restricted epitopes from the same extracellularly added precursor peptide complexed to HSP. Given previous reports of the strong immunogenicity of HSP/peptide complexes, the present data suggest that HSP-complexed peptides containing multiple MHC class I- and class II-restricted epitopes represent potential vaccine candidates for HIV and other viral infections suitable to induce effective CTL memory by simultaneously providing CD4 T cell help. *The Journal of Immunology*, 2004, 173: 1987–1993.

A robust cellular immune response appears essential for controlling HIV infection, and specific CD8^{+} and strong CD4^{+} T cell responses have been associated with better clinical outcomes in HIV-1-infected individuals (reviewed in Ref. 1). Hence, recent vaccine approaches have focused on inducing cellular immune responses mediated by CD8^{+} as well as CD4^{+} T cells (2). This need for CD4 T cell help in the induction phase of CTL responses is highlighted most clearly by recent reports in murine systems describing the induction of functionally impaired memory CTL when CD4 T cell responses were not induced simultaneously (3, 4).

Vaccination with tumor and viral Ags complexed to heat shock proteins (HSP)[4] induces strong immunity to tumors and viral infections in the murine model (5, 6), suggesting that these agents may be useful in vaccine development. HSP are the most abundant soluble intracellular proteins and are expressed at high levels during cell stress resulting from exposure to heat, toxins, glucose deprivation, and oxidative conditions (reviewed in Ref. 7). In early studies, HSP isolated from murine tumor cells were shown to confer protection to HSP-vaccinated animals against tumor challenge (8), and subsequent studies revealed that the immunogenicity of

HSP is due to their ability to associate with and carry cellular peptides, among them tumor-specific Ags (9). Upon cell stress and cell death, such HSP/Ag complexes are released and taken up by professional APCs through HSP receptors such as CD91, enabling them to cross-present Ag-derived CTL and Th cell epitopes (7, 10, 11). Importantly, not only tumor-derived Ags but also viral protein-derived peptides can induce protective immunity (12, 13), and human vaccine trials aimed at inducing both tumor- and virus-specific immunity are currently in progress (14–16). Interestingly, a number of studies have shown that HSP/peptide complexes can lead to Ag presentation on both MHC class I and class II molecules, thus activating CD8^{+} CTL as well as CD4^{+} Th cells (17–19). However, no study has addressed the presentation of HLA class I and class II epitopes from short peptides containing multiple overlapping CTL and Th epitopes, nor has the presentation of HSP complexed to HIV-derived Ags been examined (20, 21).

In the present work we took advantage of the uniquely well-characterized CTL and Th epitope landscape of HIV proteins and identified a region in HIV Gag p24 that contains overlapping CTL and Th epitopes (22) to study the simultaneous presentation of these epitopes from a single precursor peptide complexed to HSP gp96. Our data demonstrate efficient HSP-mediated presentation of at least eight different CTL and Th epitopes from the same precursor peptide sequence. These results show that HSP constructs containing CTL and Th epitopes hold potential as vaccine candidates for inducing robust cellular immunity against viral infections, particularly HIV, for which a number of clustered CTL and Th epitopes have been described (14, 16, 22).

Materials and Methods

Target cells, T cell clones, and epitope specificity

Human EBV-transformed B cell lines (B-LCL) were generated as described previously (23) and cultured in R20 medium (RPMI 1640 (Sigma-Aldrich, St. Louis, MO), 20% heat-inactivated FCS (Sigma-Aldrich), 10 mM HEPES buffer (Sigma-Aldrich), and antibiotics (2 mM L-glutamine and 50 U/ml penicillin-streptomycin)). Immature human dendritic cells (DC) were generated by culturing the plastic adherent fraction of PBMC with 150 ng of GM-CSF (R&D Systems, Minneapolis, MN), 60 ng of IL-4,

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and 1% autologous plasma (supplemented with L-glutamine, penicillin-streptomycin, and HEPES buffer) as previously described (24). The cytokines were added to the cultures on days 0, 2, and 4. On day 5 or 6, nonadherent cells were transferred to new plates, and monocyte-conditioned medium was used to mature DC (24). CD8$^+$ and CD4$^+$ T cell clones were generated and cultured as previously described (25). The epitope specificity and HLA restriction of these clones and lines have been shown previously and included clones directed against epitopes within the selected 32-mer epitope as well as control clones specific for CTL epitopes outside this region of the HIV Gag protein (26-28). The sequence of the 32-mer peptide was based on the HXB2 HIV-1 sequence (GenBank accession no. K03455) and included residues Gag p24 29-60 (EEKAFSPEVIPMFSALSEGATPQDLNTMLNTV).

FACS analyses

Surface staining of T cell clones, B-LCL, and mature and immature DC was performed by incubating 10^5 cells for 30 min at 4°C with mAbs directed against the HSP receptor CD91 (PROGEN Biotechnik, Heidelberg, Germany), and CD14, CD83, CD86, or HLA class II (all from BD Biosciences, Mountain View, CA). After washing cells with PBS/1% FCS and fixing using PBS/1% FCS/2% paraformaldehyde, samples were analyzed on a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences).

ELISPOT assays

In initial experiments, HSP/complex and 32-mer peptide concentrations between 0.0055 and 10 μg/ml soluble 32-mer were tested (29, 30). Ag concentrations representing minimal concentrations of the complex and the 32-mer required to induce weak positive responses in most clones tested were then selected for the experiments shown (29, 30). Briefly, APCs (B-LCL or DC) were incubated in 250–300 μl of medium (R10 or serum-free medium) for 3–5 h at 37°C with Ag at the following concentrations: 1) HSP gp96–32mer p24 peptide complex at 156 μg/ml, 2) HSP gp96–32mer p24 peptide complex at 56 μg/ml, 3) HSP gp96 alone at 156 μg/ml, 4) HSP gp96 alone at 56 μg/ml, 5) 32-mer p24 peptide alone at 0.33 μg/ml, 6) 32-mer p24 peptide alone at 0.11 μg/ml, 7) optimal peptide at 10 μg/ml, and 8) no Ag. After Ag pulse, cells were washed three times with R10 medium and used as APCs in ELISPOT assays at 50,000–100,000 cells/ well. Effector T cells were washed three times in R10 medium to remove IL-2, and then CTL were plated at 5,000 -20,000 cells/well and Th cell clones at 100 cells/well in 96-well polyvinylidene plates (Millipore, Bedford, MA) precoated with 0.5 μg/ml anti-IFN-γ mAb, 1-DIK (Mabtech, Nacka, Sweden). The plates were incubated overnight at 37°C in 5% CO$_2$ and developed as described previously (31). The number of spots was counted using the AID EliSpot Reader Unit (Autoimmun Diagnostika, Strasburg, Germany), and results were expressed as spot-forming cells per million input cells. The cutoff for positive responses was 1) at least five spots per well and 2) values above background (no Ag) plus 3 SD of background wells.

Cytotoxicity assay

Target cells were pulsed with Ag in 200–400 μl for 3–5 h as indicated. They were then labeled with ^{51}Cr (Na$_2$CrO$_4$, 50 μCi; NEN, Boston, MA) for 1 h at 37°C in 5% CO$_2$. Targets were washed three times before being added to effector T cells in 96-well, round-bottom plates, with each well containing 5,000 -10,000 target cells and 30,000 -100,000 effector T cells (E:T ratios between 3:1 and 10:1). After a 4- to 6-h incubation at 37°C in 5% CO$_2$, 30 μl/well of supernatant was assayed for released ^{51}Cr. Assays were set up in triplicate wells in all experiments. Maximal release was obtained by adding 100 μl of 5% Triton X-100 to target cells. The percentage of specific ^{51}Cr release was calculated as the ratio 100 × (cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release). For Ag processing inhibition experiments, target cells were incubated with the indicated concentration of inhibitor (15–75 μM brefeldin A (Sigma-Aldrich) and 25–75 μM lactacystin (ICN Biomedicals, Costa Mesa, CA)) during the Ag and ^{51}Cr pulses. The drugs were also present at the indicated concentrations throughout the assay, including the washing steps and the 6-h incubation period.

Purified murine HSP gp96 and HSP/peptide complex formation

Purification of HSP has been described previously (17, 32). Mouse organs were homogenized in a 30 mM Na$_2$PO$_4$/1.5 mM MgCl$_2$ buffer using 0.1 M AEBSF as a protease inhibitor and were centrifuged at 18,000 × g for 20 min. The supernatant was again spun at 100,000 × g for 90 min before being precipitated with 50% ammonium sulfate saturation at 2.14 g/min and incubated for 45 min. The solution was then centrifuged at 18,000 ×

g for 20 min, and supernatant was precipitated with 80% ammonium sulfate saturation at 1.82 g/min and incubated overnight for 18 h before being centrifuged at 18,000 × g for 20 min. The pellet was resuspended in PBS, 2 mM MgCl$_2$, and 2 mM CaCl$_2$ at 5 ml/g of starting tissue. The resuspended pellet was subsequently loaded onto a gravity-feed Con A column and eluted with PBS with 2 mM MgCl$_2$, 2 mM CaCl$_2$, and 10% α-D-mannopyranoside buffer. The eluent was loaded onto a gravity-feed DEAE column and eluted in 10 mM Na$_2$PO$_4$/700 mM NaCl buffer. Protein analysis of the fractions was performed by Bradford analysis, and protein-containing fractions were pooled and stored at −80°C (17). For complex formation, the HIV-1 Gag p24-derived 32-mer peptide (98% purity) was reconstituted in sterile deionized H$_2$O at a concentration of 2 mg/ml and mixed with gp96 at a 50:1 molar excess of peptide to gp96 (i.e., 3000 μg of gp96 was combined with 5417 μg of p24 32-mer peptide) by placing the tube in a 37°C water bath for 30 min (17). The complex was then washed using a Millipore spin column (UFV4BTK00) with a 30,000 m.w. cutoff and 5 column volumes of PBS. The final concentration of HSP/peptide complex was determined by Bradford protein analysis. Initial experiments were performed using ^{125}I-labeled 32-mer peptide to verify that no radioactive counts (representing free, uncomplexed peptide) remained in the spin column flow (data not shown).

The efficiency of peptide loading on gp96 was determined, based on the methods of Blachere et al. (17). Briefly, the radioactivity in 1 μg (1.76 × 10^{14} molecules) of ^{125}I-labeled 32-mer peptide (m.w., ~3,520) was determined to be 518,060 cpm by counting in a gamma counter. After gp96 was complexed to the ^{125}I-labeled peptide (50:1 molar excess of peptide to protein) and washed to remove unbound material, 1 μg of gp96 (6.46 × 10^{12} molecules) was run on SDS-PAGE. The band migrating at 96,000 Da was cut from the gel and counted in a gamma counter, yielding 1,083 cpm. This count corresponds to 3.68 × 10^{11} molecules of peptide, or ~5% loading efficiency. The complexing reaction, SDS-PAGE, and determination of radioactive counts in the gp96 band were performed a total of three times, yielding similar gp96 loading efficiencies. The quantity of free peptide (0.33 μg/ml) used as a negative control in re-presentation assays corresponds to the amount of peptide present in 156 μg/ml peptide-complexed gp96, assuming the 5% loading efficiency described above.

Results

Selection of HIV Gag p24 peptide EV32

HIV-specific T cell responses have been intensively studied over the last 15 years, and >200 CTL and Th cell epitopes have been optimally defined to date (22, 33). Many of these responses cluster and may reflect regions of the viral genome that are relatively conserved and efficiently processed and presented by both HLA class I and class II molecules (34–36). To address whether HSP could simultaneously present multiple epitopes contained in a longer peptide, we studied a unique region of clustered CTL and Th epitopes within HIV Gag p24 (residues 29–60, EEKAFSPE-VIPMFSALSEGATPQDLNTMLNTV, termed peptide EV32) that are restricted by multiple class I and class II alleles and for which an exceptional selection of specific T cell clones was available in our laboratory. Well-defined epitopes within EV32 include those restricted by HLA-A26, -B7, -B42, -B44, -B57, -B53, -B58, -B60, -Cw1, and -Cw8 (28). In addition, class II epitopes restricted by HLA-DR01013, -DR0404, and -DQ0503 have been identified (Fig. 1) (29). Thus, EV32 represents an ideal candidate to assess simultaneous presentation of epitopes on HLA class I and class II molecules. Furthermore, due to the high frequency of at least some of the HLA alleles restricting epitopes in this region, peptide EV32 could also represent a preferred candidate for HSP-based HIV vaccination trials, because it may be suitable to induce responses largely independent of the individual's HLA type (36).

Expression of CD91 on APC

Immature DC of the myeloid lineage have been shown in separate studies to represent antigenic epitopes from HSP/peptide complexes on MHC class I and class II molecules (reviewed in Ref. 7). Initial presentation studies were therefore conducted using immature human DC from a number of donors expressing some of the

FIGURE 1. Localization of HLA class I- and class II-restricted T cell epitopes in the 32-mer EV32 peptide from HIV-1 Gag p24. Only epitopes that were included in the present study are indicated (see Ref. 26 for full set of epitopes located in this region).
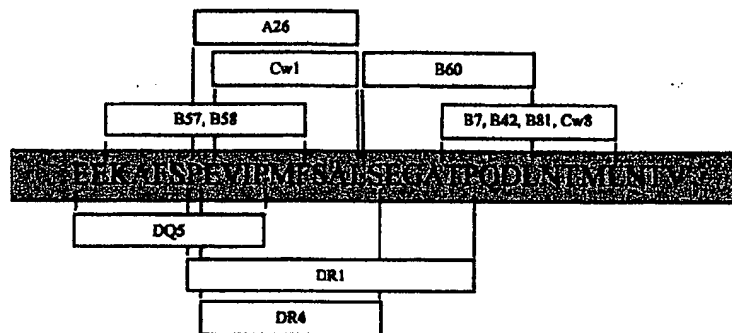
above HLA alleles. However, when ELISPOT assays were performed using HSP or HSP-p24 complexes. high background values were observed (data not shown). This background may have been due to nonspecific activation signals or cytokine release from DC because the exposure of immature DC to HSP alone can induce maturation of DC (24, 37–39). Therefore, to facilitate the analyses and, moreover, to be less constrained by obtaining sufficient DC from individuals with the appropriate HLA types, a number of well-established cell lines and in vitro generated B-LCL were assessed for the expression of CD91, a common receptor for HSP (10). B-LCL were found to express significant levels of CD91, whereas other cells, such as T cell clones and cell lines such as RAJE and BJAB, were CD91 negative. The staining intensity of the B-LCL was similar to that of immature DC (Fig. 2). These data provide the first demonstration of CD91 expression on human B cells, at least upon EBV transformation.

In this study, finding CD91 expression on B cells was also important from a practical point of view. DC require a complex process for growing and culture and can only be used in assays over a 2- to 3-day period. In contrast, B-LCL can be kept in culture for an extensive period and are easy to handle, which makes them ideal target cells and APC in the assays described in this study. Furthermore, very little background was observed in ELISPOTs when using B cell lines instead of immature DC.

*Presentation of HSP-complexed HIV-1 Gag p24 32-mer peptide*

Presentation of the HSP-p24 complex was first examined in ELISPOT assays using HLA class I-restricted CTL and class II-restricted Th clones in separate experiments. With an HLA B57-restricted CD8⁺ CTL clone and HLA B57-expressing B-LCL as APC, strong responses to the HSP-p24 complex were consistently detected, whereas HSP alone or the soluble 32-mer peptide alone did not induce significant responses (Fig. 3A). The responses to the

FIGURE 2. CD91 expression on B-LCL and immature dendritic cells. Solid lines indicate staining intensity using an anti-CD91 mAb; lighter lines indicate staining using an isotype control.

known optimal peptide targeted by this clone (KAFSPEVIPMF) and to PHA served as positive controls to assure activity and specificity of the effector T cells. Importantly, the peptide molarity of the 32-mer alone was equal to the peptide molarity in the HSP/peptide complex and was based on the calculated complex formation efficiency as described in *Materials and Methods*. The absence of a significant response against the 32-mer alone helped to rule out the possibility that contaminating, unbound 32-mer peptide in the HSP complex preparation was responsible for the responses detected with the HSP/peptide complex. Furthermore. when no APC were used, and Ag was added directly to the effector T cells, no responses were observed against the HSP/peptide complex (data not shown). This lack of response suggests that no significant amount of contaminating soluble peptide was present in the complex solution and that HSP receptor expression by the APC was required for re-presentation. Similar results were obtained using an HLA-DQ5 restricted-CD4⁺ T cell clone and HLA-DQ5-expressing B-LCL as target cells in ELISPOT assays (Fig. 3B). As with CTL, no responses were detected to HSP alone, and weak responses were seen to the 32-mer peptide, which could reflect direct binding of the 32-mer, exogenous peptide to the class II molecules. Of note, the HSP/peptide complex at 156 μg/ml elicited the same magnitude of response as the optimal epitope described for this clone despite a 30 times lower peptide molarity in the complex compared with the optimal epitope, suggesting that cross-presentation may occur with high efficiency. Additionally. no responses were seen when a CD4⁺ T cell clone specific for a different region of Gag was stimulated with either the HSP/32-mer complex or 32-mer alone (data not shown).

To test whether stimulation with the HSP/peptide complex elicited more T cell effector functions beyond IFN-γ secretion, cytotoxicity assays using HLA-matched and mismatched targets cells pulsed with Ag and ⁵¹Cr were performed. Additionally, the Ag processing inhibitory effects of brefeldin A could be studied in cytotoxicity assays, but not in ELISPOT assays, where brefeldin A impairs the secretion of IFN-γ. Target cells were pulsed and extensively washed before effector T cells were added to the assay. thus removing any potentially contaminating uncomplexed 32-mer from the assay. Recognition of CD4⁺ T cell epitopes on class II molecules was also assessed in cytotoxicity assays (Fig. 3C), taking advantage of the cytotoxic potential of these CD4⁺ clones (27, 40). In assays using an HLA-B42-restricted, CD8⁺ CTL clone and HLA-B42-expressing B-LCL, strong cytolytic activity was observed against targets pulsed with the optimal peptide and the HSP/peptide complex, whereas targets pulsed with HSP gp96 or soluble 32-mer peptide alone were not lysed above background levels (Fig. 3D). An HLA-B60-positive, HLA-B42-negative B-LCL included in the same assay was not recognized by the
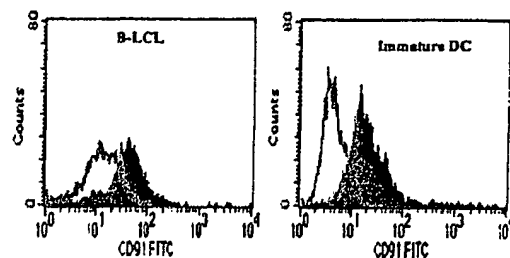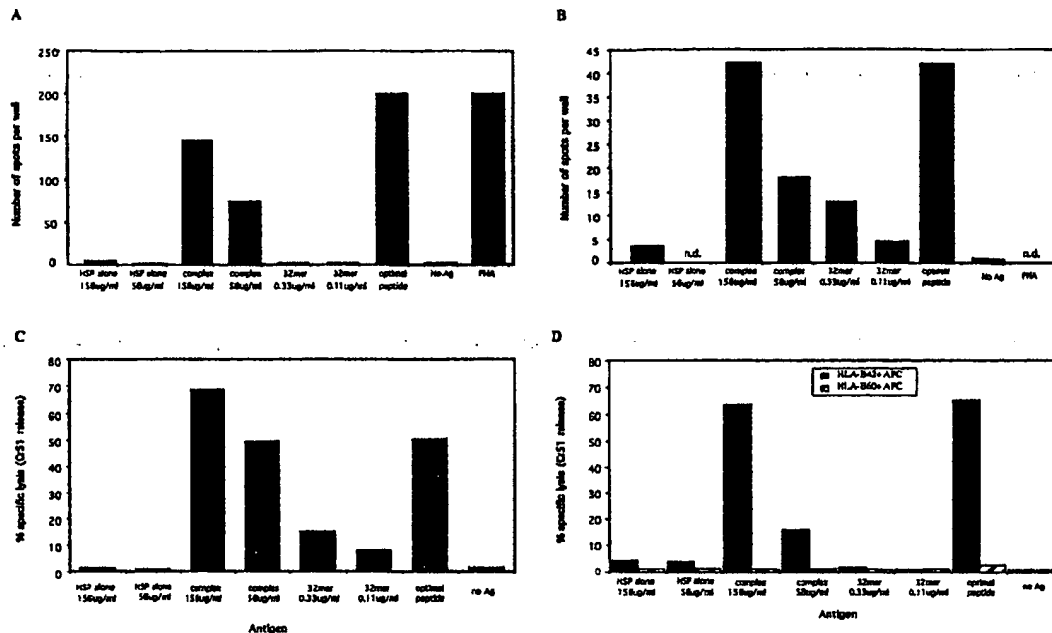
FIGURE 3. Presentation of class I and class II restricted epitopes. *A*, ELISPOT assay: HLA-B57-expressing B-LCL were pulsed with different Ags and incubated with HLA-B57-restricted CD8⁺ CTL clone. The Ag concentrations used were as indicated, and the optimal epitope was KAFSPEVIPMF. *B*, ELISPOT assay: DQ5-expressing B-LCL presented Ag to a DQ5-restricted CD4⁺ T cell clone at the indicated concentrations. The optimal epitope for this clone was EEKAFSPEVIP. *C*, ⁵¹Cr assay: presentation of a class II-derived epitope assessed in cytotoxicity assays. The same clone as in *B* was used in a standard ⁵¹Cr release assay at an E:T cell ratio of 10:1 and using the autologous, HLA-DQ5 expressing B-LCL as target cell previously pulsed for 4 h with the indicated Ags. *D*, HLA dependence of re-presentation. HLA-B42⁺/B60⁻ and B42⁻/B60⁺ B-LCL were used as target cells in a ⁵¹Cr release assay using a HLA-B42-restricted CTL clone. E:T cell ratio was 10:1 and the optimal peptide (TPQDLNTML) was used at 10 µg/ml. Specific lysis was calculated from ⁵¹Cr released after 4-h incubation. One representative experiment of three to five independent experiments is shown in all figures.

HLA-B42-restricted clone, demonstrating that these responses were HLA restricted and epitope specific. In addition, presentation was achieved for other T cell epitopes located within the EV32 peptide that were restricted by HLA-B7 (TPQDLNTML), HLA-B60 (SEGATPQDL), Cw8 (TPQDLNTML), as well as HLA-DR0404 (EVIPMFSALS) and DR0102 (PEVIPMFSALSEGATP; Fig. 1 and data not shown). Taken together, these data demonstrate that multiple HLA class I-restricted (5) and class II-restricted (3) epitopes can be processed and presented from the 32-mer precursor peptide coupled to HSP and that for re-presentation of Ag, HSP receptors may be required.

*Simultaneous presentation on HLA class I and class II*

Additional experiments were conducted to address whether HIV epitopes in the HSP/peptide complex could be re-presented concurrently by HLA class I and class II molecules on the same target cells. To this end, target cells expressing both HLA-B57 and DQ5 alleles were incubated separately with an HLA-B57 CTL and HLA-DQ5 Th clone as effector cells in a ⁵¹Cr release assay. Both the B57-restricted CD8⁻ CTL and the DQ5-restricted CD4⁺ cells recognized and lysed the HSP-p24 complex-pulsed B-LCL in the same experiment, although the level of lysis by the B57-restricted CTL clone was lower than that induced by the DQ5-restricted clone (Fig. 4). However, the difference in magnitude of killing probably reflects variable cytolytic activity of the clones rather than differences in the presentation efficiency between class I and class II peptides, because the same differences in killing were ob-

served when target cells were pulsed with saturating concentrations (10 µg/ml) of their respective optimal epitopes.

Ag re-presentation on HLA class I is inhibited by brefeldin A, but not by lactacystin

HLA class I molecules generally present CTL epitopes derived from newly synthesized, intracellular proteins. After digestion by the proteasome, the processed peptides are translocated to the endoplasmatic reticulum by the TAP heterodimer (41). However, numerous exceptions to this rule have been reported, and evidence for MHC class I-restricted presentation of exogenous Ag has existed for several years (42–45). To address which processing pathways were used for the presentation of CTL epitopes derived from the HSP-bound EV32 peptide, the sensitivity of these pathways to a number of processing/presentation inhibitors was examined (43, 46). Fig. 5*A* summarizes data from experiments in which two different processing inhibitors were present. Brefeldin A, an inhibitor of post-Golgi transport and thereby an inhibitor of HLA class I-restricted Ag presentation (47, 48), reduced lysis of HSP-p24-pulsed, but not optimal peptide-pulsed, B-LCL by almost 70%. In contrast, lactacystin, a proteasomal inhibitor, showed only a weak inhibitory effect at the highest concentration tested, suggesting that other mechanisms besides proteasomes are involved in the processing of the HSP-p24 complex (49). No effect of brefeldin A on HLA class II-restricted presentation was noted in experiments in which a CD8⁺ and a CD4⁺ T cell clone were tested side by side on the same target cells expressing appropriate class I and class II

FIGURE 4. Presentation of the complex to CTL and Th cell clones using the same APC: an HLA B57/DQ5-expressing B-LCL (HLA A24/31, B14/57, Bw4/6, C7/8, DR1/4, DRw53, and DQ3/5) was incubated with Ags at 156 μg/ml (HSP/p24 complex), 0.33 μg/ml (32-mer peptide), and 10 μg/ml (optimal epitope); ^{51}Cr labeled; and incubated separately with an HLA-B57-restricted CTL clone (derived from a HLA-A2,-30; B44,-57; C8,-18; DR8,-13, DQ1,-4 allele-expressing donor) or a DQ5-restricted Th clone (from a HLA-A2,-3; B35,-55; DR11,-14; DQ3,-5 allele-expressing donor). The percent specific lysis is shown after 4-h incubation and represents average killing and SD.
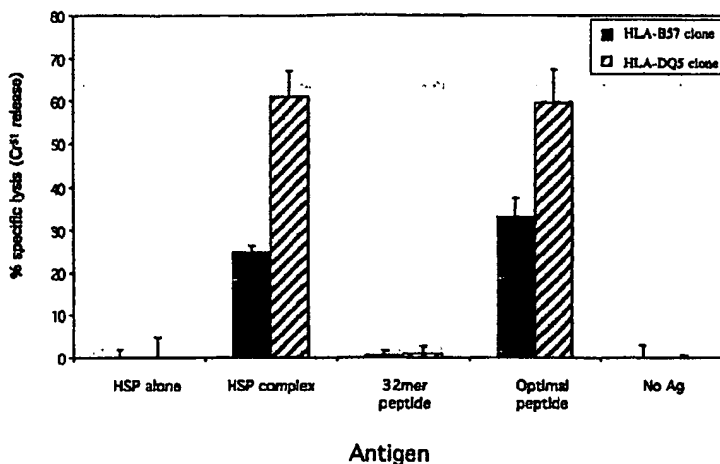

alleles (Fig. 5B). This implies that class II re-presentation of HSP/peptide complex-derived epitopes does not depend on newly synthesized class II molecules. In addition, given the high level of combined killing by CD4 and CD8 T cells of the same target cell population (>90%), the data strongly suggest that at least some of the target cells simultaneously processed and presented both HLA class I- and class II-restricted HIV epitopes efficiently.

Discussion

The data presented in this report are in agreement with the results of several earlier studies that have shown presentation of exoge-nous Ag on HLA class I by TAP-dependent and independent pathways (10, 43, 46, 50–52). We previously reported that a carrier-peptide complex (composed of an influenza matrix CTL peptide covalently bound to transferrin) was also presented by a brefeldin A-sensitive endogenous Ag processing pathway (43). In more recent studies using HSP as vehicles for Ags, others have found that the presentation of HSP70-chaperoned peptides may be processed through a nonendosomal, endoplasmatic reticulum-dependent route (50). Interestingly, depending on the C-terminal sequence of the class I epitope, an alternate processing mechanism may exist that is lactacystin resistant and endosome dependent (50). Whether

A

B
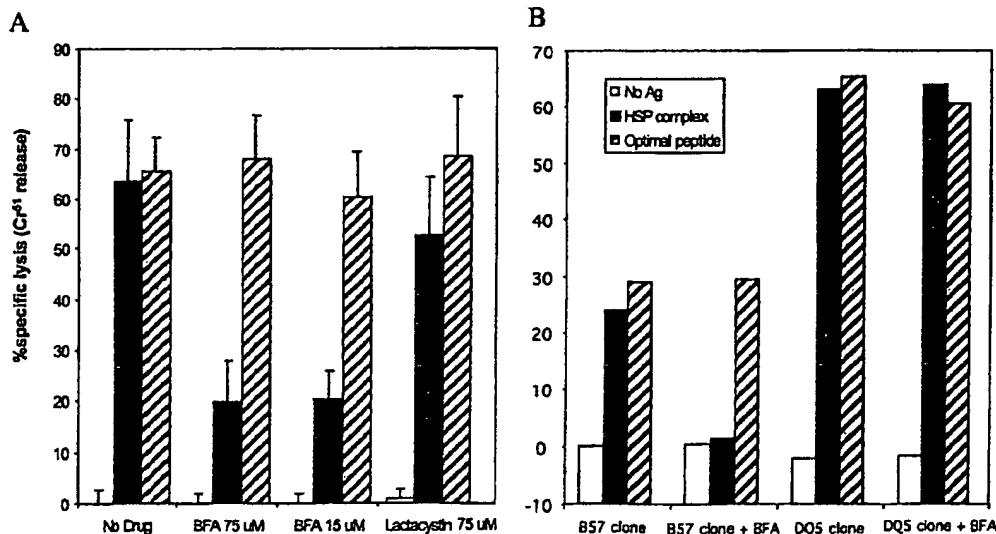
FIGURE 5. Effect of inhibitors of Ag processing on re-presentation. The average and SD of three independent experiments is shown in A where inhibitors were present during the entire assay at the concentrations indicated. Ag-presenting B-LCL were pulsed with Ag and ^{51}Cr as described in Fig. 4. Specific lysis was determined from released ^{51}Cr after a 4-h incubation of HLA-B42-expressing target cells with an HLA-B42-restricted CTL clone. Effective doses of lactacystin were used, as determined in parallel experiments where processing and presentation of the HLA-B57 restricted epitope from HIV-Gag expressing, recombinant vaccinia virus constructs were inhibited by 80% at 75 μM (data not shown). B, One representative experiment is shown in which an HLA-B57-expressing and an HLA-DQ5-expressing target cells line were incubated with no Ag, HSP/peptide complex, or the optimal epitope for the B57- and DQ5-restricted T cell clones, respectively. The assay was performed with target cells incubated with or without 75 μM brefeldin A. Complex recognition was only inhibited for the HLA-B57-restricted but not the HLA-DQ5-restricted, clone. Inhibition of HLA class II-restricted clones was tested in two additional independent experiments with the same outcome.

other HIV-1 peptides with different sequences can be processed via an endosomal route remains to be addressed.

Although we have not assessed the processing pathways for all epitopes contained in the 32-mer peptide in this study, the data clearly show that multiple epitopes (at least five CTL and three Th cell epitopes) can be efficiently processed from the 32-mer precursor peptide. The present study provides the first demonstration of HSP-mediated, concurrent HLA class I- and class II-restricted representation of Ag, taking advantage of the unique regions in HIV in which multiple class I- and class II-restricted epitopes overlap. This type of overlap may exist in other pathogens as well, but such constellations have not yet been identified due to limited knowledge of T cell targets in these pathogens. It has also been postulated that simultaneous presentation of class I- and class II-restricted epitopes by the same cell may be required for successful induction of CTL (53). The present HSP complex would probably fulfill this requirement, and vaccine candidates for other pathogens could be engineered to deliver CTL and Th epitopes to the same APC.

A potential limitation of the present study is that all experiments reported in this study were conducted in EBV-transformed B cell lines. Although experiments using DC as APC were inconclusive due to high background problems, it is unlikely that DC would process Ag less efficiently than B cell lines; therefore, it may be expected that efficient presentation on HLA class I and class II could occur on DC as well (39, 54–58). In addition, recent findings documenting the expression of HSP receptors on peripheral blood B cells and, especially, increased CD91 expression on monocytes from HIV infected long term nonprogressors point to the relevance of B cells and monocytes in binding and possibly re-presenting Ag in vivo and thus underline the significance of the model used in the present study (54, 59, 60). Similarly, the induction of Ag-specific T cell responses upon HSP-Ag immunization in other viral systems and in cancer patients provides strong support that the mechanism described in the present study could be active in vivo (14, 61, 62). Thus, the ability of an HSP-HIV peptide complex to activate CD8$^+$ and CD4$^+$ T cells in vitro bears considerable potential for future clinical applications, especially with recent reports pointing out the essential role of costimulated CD4 responses in the induction of functionally competent memory CTL (3, 4). The concurrent stimulation of CD8$^+$ and CD4$^+$ T cells demonstrated in the present report opens up the possibility of designing simple vaccines that activate both branches of the T cell response by using a single HSP/peptide complex containing class I- and class II-restricted epitopes. Furthermore, given the clustering of overlapping CTL epitopes restricted by many different HLA alleles, a short region of the viral genome may provide vaccine candidates for diverse HLA backgrounds (36). Alternatively, for whole genome vaccine approaches or single-epitope vaccines, HSP purified from HIV-infected cells could be used as the Ag source for a largely HLA-independent vaccine able to induce CTL and Th cell responses. Both approaches hold promise for strengthening the cellular immune response in HIV patients and potentially in HIV-uninfected vaccinees; the findings presented in this study clearly warrant investigation of the immunogenicity and usefulness of HSP/peptide complexes as HIV vaccines in well-controlled clinical trials.

Note added in proof. Since the submission of this manuscript, a related paper has been published (63) showing that gp96 can also chaperone MHC class I and class II epitopes for in vivo presentation.

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